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Łukasz Jurczyk, PhD, Eng., Professor at the University of Rzeszów
University of Rzeszów
Faculty of Technology and Life Sciences
Institute of Agricultural Sciences, Environmental Protection and Management
35-601 Rzeszów, ul. Ćwiklińskiej 1A
tel. 17 872 17 25, e-mail: ljurczyk@ur.edu.pl

REVIEW

of the doctoral dissertation by Ruchi Manishkumar Upadhyay, M.Sc. entitled “Determination of the mechanism and optimisation of the conditions of the process of removing colored aromatic compounds by selected Basidiomycota"

1. Subject and formal basis of the review

The subject of the review is the doctoral dissertation entitled “Determination of the mechanism and optimisation of the conditions of the process of removing coloured aromatic compounds by Basidiomycota”, authored by Ruchi Manishkumar Upadhyay, M.Sc. and supervised by Prof. Wioletta Przystaś, PhD, Eng.

The formal basis for the preparation of this review is the resolution of the Discipline Council for Environmental Engineering, Mining and Power Engineering at the Faculty of Energy and Environmental Engineering, Silesian University of Technology, dated 19 February 2026, and the cover letter (RIE-BD.512.14.2026) from the Chair of the Council, Prof. Krzysztof Labus, dated 3 March 2026, which was received, together with a printed copy of the dissertation, at the Registry of the University of Rzeszów on 12 March 2026.

2. Justification for the topic – description of the theses, objectives and scope of the work

In Chapter **3, ‘Research Statement**’ (p. 19), the author, based on the preceding literature review, posits that the methods currently used to remove synthetic dyes from industrial wastewater are insufficient. In her view, an effective solution would be the introduction of decentralised, compact and rapid treatment systems that could operate directly at the source of pollution. Such systems could be based on biological reactors containing Basidiomycota fungal biomass, which would constitute a sustainable alternative to other treatment methods. In this context, she identified five areas of knowledge where there are gaps of critical importance to the functioning of such technologies; (1) firstly, insufficient understanding at the molecular and biochemical levels of the mechanisms by which fungi remove pollutants through biosorption and biodegradation, (2) secondly, a lack of comprehensive research into the optimisation of technological parameters for different fungal species, with a view to increasing treatment efficiency, (3) thirdly, a limited multi-omic approach in existing research, which would allow for a fuller understanding of the metabolic pathways involved in the degradation of specific dyes, (4) a further issue is the lack of sufficient toxicity assessment of treatment products, particularly using standardised tests, (5) finally, the last point mentioned is the failure to translate existing laboratory-scale research results into engineering systems for the treatment of synthetic dyes at a scale appropriate for decentralisation. On this basis, the author formulated the following research hypothesis: *white rot fungi, such as T. versicolor and P. ostreatus, demonstrate the ability to remove dyes through a synergistic mechanism of biosorption and enzymatic biodegradation, which can be significantly improved by optimising key physicochemical parameters and effectively applied in compact, decentralised bioreactor systems designed for specific dyes. Treatment can lead to a reduction in effluent toxicity, whilst a multi-omics approach can help to understand the relationship between enzyme activity and gene expression, which may form the basis for modelling interactions between these enzymes and various classes of dyes*.

The author then outlined the **scope of research** (Fig. 2) required to confirm this hypothesis, focusing on three areas: (1) **optimisation of the treatment process** (including species selection, methods of utilising their biomass and optimisation of key technological parameters using advanced statistical and modelling tools), **identification of degradation mechanisms** (using a multi-omics approach) and **reduction of toxicity** (confirmed by studies on selected model organisms). Consequently, Ms Ruchi Manishkumar Upadhyay systematically formulated a series of detailed **research objectives** in her dissertation, which are as follows: (1) **Optimisation of biodegradation parameters**, *in particular physicochemical parameters, the role of immobilisation, biomass and dye concentrations, exposure time, agitation, carbon and nitrogen sources, and lighting conditions*, (2) **optimisation of biosorption parameters**, *in particular physicochemical parameters, the effect of immobilisation, biomass condition, agitation, dye concentration, exposure time, pH and temperature*, (3) **multi-omics analysis**, *in particular analysis of the transcriptome, proteome and enzyme profile*, (4) **assessment of the dye removal mechanism**, *in particular assessment of the contribution of degradation and sorption, analysis of the biosorbent's functional groups, and derivation of the dye removal mechanism based on Gene Ontology (GO*), (5) **Ecotoxicological assessment***, in particular a comprehensive assessment of the impact of purification products on environmental safety, determination of differences in zootoxicity towards D. magna and phytotoxicity towards Spirodela polyrhiza of pure dyes and dyes after the purification process,* and (6) **bioreactor design concept**; development of bioreactor specifications based on optimised parameters.

In the introduction and literature review, the PhD candidate provided a thorough justification of the scale of the problem that prompted her to undertake this research. The current globalisation of the economy means that the burden of textile production has long since shifted to South and East Asia. It must be emphasised, however, that EU countries, among others, are one of the main consumers of these textiles, effectively shifting the consequences of environmental pollution beyond their own borders, but also the responsibility and control. At the same time, in that cultural region, the significance of dyes extends far beyond aesthetics; it also concerns traditions, beliefs and identity, and the history of their use in dyeing fabrics dates back at least several thousand years.

The completion of the research tasks set out in the dissertation was an ambitious goal and required, above all, a comprehensive research methodology, spanning many fields and involving specialist analytical techniques, comprising consistently well-thought-out and systematically conducted experiments leading to the acquisition of a broad spectrum and significant quantities of data for statistical analysis.

3. General characteristics of the doctoral dissertation – structure and content

The doctoral dissertation by Ruchi Manishkumar Upadhyay, M.Sc., submitted to me for review, has been prepared as a single monograph written in English, totalling 204 pages, including formal elements and appendices. The dissertation follows the standard structure of a scientific monograph corresponding to a doctoral dissertation in the field of engineering and technical sciences, based on empirical data; with a clear division into a theoretical section, defining the research hypotheses and objectives (a total of 23 pages), and an empirical section (a total of 150 pages), describing the method used to obtain the results and their discussion, concluding with findings and future research prospects.

The text of the dissertation has been enriched with 63 colour graphics in the main body (and additional ones in the appendices), including not only classic charts illustrating scientific data, but also flowcharts systematising the tasks defined, the execution of individual experiments, drawings of chemical structures and SEM images. Furthermore, the work contains 19 tables in the main body and 5 in the Appendices. From experience, I know that the use of colour graphics, particularly in the case of charts presenting detailed data, is not always clear to everyone. However, in this case, it corresponds to the subject matter of the dissertation; moreover, the colours do not appear to have been chosen at random, but rather reflect the dyes described in the dissertation (as can be seen, for example, in the decolourisation efficiency graphs on pages 61–67). This subtle touch appeals to the reader's imagination. Nevertheless, some of the figures may be difficult to read due to the large number of small labels (e.g. Fig. 43). However, the aesthetics of the work and the careful editing deserve special mention.

The dissertation begins with **a formal section**, in which the reader will find, following the title page, the **Acknowledgements**, the **Table of Contents** and **the Abstract** in English, comprising 686 words (i.e. 4769 characters excluding spaces), followed by eleven keywords (i.e.: mycoremediation; white rot fungi (note: in the Polish version, this should perhaps simply be 'white rot fungi' [*grzyby białej zgnilizny*]); synthetic dyes; biodegradation; biosorption; transcriptomics; proteomics; ecotoxicology; wastewater treatment; Trametes versicolor; *Pleurotus ostreatus*), and on the following page its translation into Polish. The abstract is quite extensive, which, however, is due to the large number of topics covered in the work, but most importantly, it is well-structured and clear. On page XII there is a list of four publications from years 2022–2025, with a combined Impact Factor (IF) of 7.7 and 340 points on the Ministry of Science and Higher Education (MNiSW) list, published by the author jointly with her supervisor. In all these publications, which relate to the subject matter described in the dissertation, Ms Ruchi Upadhyay is the first author, which highlights the mature role of the supervisor, whose task is to support the research plan and share experience in preparing publications in which the doctoral candidate plays the leading role. This type of research could not have been conducted without securing sufficient funding, in this case four projects funded by the Faculty of Energy and Environmental Engineering at the Silesian University of Technology. The formal section concludes with a list of 33 acronyms used in the dissertation. These are not all the abbreviations that can be found in the text, but they are indeed the most frequently recurring ones, e.g. the names of research variants or dyes.

The theoretical section begins with a brief, two-page **introduction** to the subject matter of the dissertation (Introduction, p. 1), in which the author notes that there is an urgent need to develop technologies for treating wastewater containing various dyes, characterised by different structures and properties – therefore, the appropriate approach is the decentralisation of treatment systems, and the technology that should be considered first as potentially the least environmentally burdensome, yet at the same time the most flexible in designing experiments to optimise treatment efficiency, is the use of biological reactors. The use of fungal biomass, specifically that of white rot fungi, appears particularly promising. The introduction, together with the literature review (p. 3, Review of literature), seems brief, as it comprises a total of only 18 pages; however, within this space, the author has managed to cite 118 references, which nevertheless demonstrates a balanced proportion between the theoretical and empirical parts of the dissertation. I would add that the reader will also find a number of references to the literature in the research methodology. The PhD candidate begins by defining the concept of dyes and briefly refers to their cultural significance and the history of their use. She then highlights, in a few sentences, the positive properties of natural dyes , setting them, as it were, in contrast to the synthetic dyes described subsequently, on which she focuses, moving on to their classification based on: chemical structure and method of application, adverse impact on the ecosystem (mainly aquatic) and human health and life – which, according to the PhD candidate, corresponds to at least several of the Sustainable Development Goals identified by the United Nations. Next, the composition of dye-containing wastewater is characterised, particularly in the context of average concentrations and legal limits in selected countries. In the following sub-section (p. 10, 2.6), the author moves on to a detailed description of methods for treating dye-containing wastewater; firstly physical methods (adsorption and membrane techniques), chemical methods (here she mentions coagulation, electrocoagulation, advanced oxidation processes and photocatalysis) and finally biological methods using biomass from bacteria, algae, yeasts and filamentous fungi. The author then moves on to a detailed analysis of the potential for dye biodegradation by white rot fungi (subsection 2.7, p. 14) and briefly discusses the taxonomy of species that degrade lignin in nature and the enzymatic mechanism they utilise. Subsequently, the range of chemical substances that may also be biodegradable by these species is presented – including plant protection products, heavy metals, antibiotics and anti-inflammatory or hormonal drugs, as well as PAHs, PCBs and, finally, dyes. The latter are removed via biosorption, bioaccumulation and enzymatic biodegradation – yet, as the PhD student emphasises, *"the full mechanism underlying bioremediation is not well understood*".

The work goes on to discuss methods of enzyme immobilisation (their advantages and disadvantages are summarised in a table), the sorption potential of dead and living fungal biomass for dyes, and the author also lists materials that could serve as potential biomass carriers, before finally moving on to the advantages of multi-omics in fungal analysis. The PhD student notes that an insufficient understanding of the complex mechanisms governing the degradation of harmful textile dyes may limit the application of biological methods. New multi-omics tools are expected to enable an integrated approach to this problem. In the remainder of the literature review, the author moves on to discuss issues related to the toxicology of treatment products – she notes that detoxification is not usually the same as decolourisation, and that degradation products may pose a potentially greater environmental risk than the original dye – hence the need to assess the toxicity of effluents from the treatment system using a battery of tests representing different trophic levels. In the summary of this chapter, the author lists the adverse factors that hinder the implementation of bioreactor technologies using fungal biomass and highlights the need to fill the knowledge gaps in order to overcome them.

The **Research Statement** chapter has been discussed above, in the description of the dissertation, its objectives and scope.

The 'Materials and Methods' section (p. 24) is very extensive, both in terms of length (35 pages) and structure – I counted as many as 47 subsections here (!). This chapter is also richly illustrated with diagrams showing the experimental set-ups for the various test variants. In the first part, the author describes the selection of chemical compounds chosen for the studies, comprising two azo dyes (Congo Red – CR and Evans Blue – EB) and triphenylmethane dyes (Brilliant Green – BG and Crystal Violet – CV), as well as one anthraquinone dye (Brilliant Blue Remazol R – RBBR), and the method of preparing their solutions used in subsequent experiments. The author then moves on to the selection, preparation and cultivation conditions for the mycelium strains – oyster mushroom (*Pleurotus ostreatus*, strain BWPH) and variegated tinder fungus (*Trametes versicolor*, CB8).

As biomass carriers, the PhD student selected materials widely available in retail outlets, namely PP scourers and PU sponges. In my opinion, slightly different terminology should be used later in the dissertation, to which I refer in the comments below.

In subsequent experiments, the biomass appears in free form, as mycelial aggregates (self-immobilised, mycelial pellets) and immobilised on PP and PU; the latter also in a non-viable form (autoclaved) and, depending on the variant, is cultured under static conditions or shaken. This experimental setup required the use of an expanded range of control samples (Fig. 10, p. 32). In order to optimise the dye biodegradation process, the influence of technological conditions such as shaken, the source of carbon and nitrogen in the culture medium, the method of biomass immobilisation, and exposure to the dye mixture was investigated.

In my view, the assessment of biological activity at successive levels of cellular organisation within the dye-removal process represents the most significant cognitive aspect of this work. Firstly, the method for isolating and assessing the activity of the main enzymes potentially involved in the degradation of the three selected dyes (RBBR, EB and CV) – laccases (Lacc), manganese peroxidase (MnP) and lignin peroxidase (LiP) – was described. For the transcriptome analysis, the *T. versicolor* strain, which showed the most promise in preliminary studies, and two dyes (RBBR and EB) were selected as factors differentiating gene expression. Extraction and quality and quantity checks of total RNA were performed at the SUT, whilst the resulting isolates were sent to a commercial subcontractor for library preparation and sequencing. However, the author of the dissertation is familiar with and describes the subsequent stages – purification, fragmentation, reverse transcription, ligation and PCR. She also describes in detail the bioinformatic processing of the data obtained from RNA-seq. For gene ontology analyses, genes with an expression change of ≥2 were selected, and comparisons of samples exposed to dyes with control samples were based on data from the NCBI gene bank.

Another dataset essential for the multi-omic approach was the proteome analysis *of T. versicolor*, which was selected due to its superior efficacy in degrading the RBBR, EB and CV dyes. Following a seven-day culture and 48-hour exposure to the dyes, a three-stage proteomic analysis (protein extraction, LC-MS, bioinformatic data analysis) was carried out to identify differences in the protein profile in response to the dyes.

The next stage of the work involved assessing the ecotoxicity of the dye degradation products, based on standardised zootoxicity tests on D. *magna* and phytotoxicity tests on *Spirodela polyrhiza* (*Lemnoideae*). An interesting experiment involved determining the effect of light of different wavelengths—white, red, blue and green—on the degradation of RBBR and EB dyes by both species of white rot fungi. The next stage of the research, in turn, aimed to determine the extent to which the fungal biomass itself — through surface adsorption, absorption and intracellular accumulation — removes dyes independently of the action of extracellular enzymes. Two types of biosorbents were prepared: in the form of mycelial aggregates and immobilised on a carrier, with enzymes washed off from 7-day-old biomass. Both live and dead (autoclaved) biosorbents were tested.

The effect of the initial dye concentration (100–400 mg/L) and contact time on the biosorption efficiency of the four variants of live biosorbents was also investigated. The absorbance of the supernatant was measured after 0, 1, 2, 4, 6 and 24 hours to determine the rate and efficiency of dye removal. Data from both parameters were analysed using PCA to identify their combined effect on the biosorption process. When analysing the effect of two key parameters (temperature and pH) to optimise biosorption, other statistical methods were also applied; Central Composite Design (CCD) and Response Surface Methodology (RSM) – to develop an equation describing the relationships between the independent variables and the system's response. The potential for multiple sorption-desorption cycles of the biomass was also assessed.

Previous laboratory tests demonstrated that mycelial aggregates and *T. versicolor* biomass immobilised on a sponge removed the RBBR dye most rapidly and effectively, therefore they were selected for further testing in laboratory bioreactors, in which four consecutive cycles of dye addition were carried out using the previously prepared immobilised biomass. After a 7-day growth phase, RBBR was introduced into two bioreactors and into abiotic controls, with four 5-day cycles of dye addition being conducted. Samples were taken after each dose to determine the initial concentration, and after 48 hours the decolourisation efficiency was assessed by calculating the percentage of dye removal relative to the control.

The most extensive part of the dissertation is the 110-page description of the results and discussion (Chapter 5, from p. 59). Firstly, Ms Ruchi Upadhyay assessed the growth of fungal biomass, which varied depending on the method of immobilisation: PU sponge ensured complete and uniform colonisation, whilst PP was colonised by only 30–40%, which required the use of a greater mass of it in the reactor. The mycelial aggregates apparently had a larger sorption surface area, which increases the efficiency of initial dye sorption and influences the final degradation efficiency. The biomass on the PU sponge demonstrated significantly higher dye removal efficiency than on PP, which, according to the author, results from the carrier's greater porosity, hydrophilicity and sorption surface area, enabling better mass transport and dye diffusion. In both carriers, efficiency increased with dye concentration, then stabilised due to saturation, with the PU sponge maintaining high efficiency over a wider concentration range. Both tested strains demonstrated a very high capacity for dye decolourisation, with the CB8 strain in every form removing over 90% of EB, CV and RBBR at all tested concentrations. The BWPH strain in the form of aggregates and on PU achieved over 80% efficiency; therefore, only these two forms of immobilisation were selected for further studies, omitting PP due to poor colonisation. The CB8 strain, in the form of mycelial aggregates and immobilised, maintained very high EB removal efficiency (95–99%) at all concentrations, which, according to the author, was due to a strong system of oxidative enzymes and good tolerance to dye stress. The BWPH strain also effectively degraded EB, although its efficiency decreased at higher concentrations, and immobilisation on a sponge significantly improved stability and resistance to dye toxicity. The CB8 strain in the form of aggregates removed CR most effectively, achieving over 90% decolourisation at higher concentrations, whilst immobilisation on a sponge reduced its efficacy. BWPH was more susceptible to dye toxicity, but immobilisation on a sponge significantly improved its efficiency (up to 85%), confirming that the carrier can alleviate the stress caused by a high dye load. Desorption experiments confirmed that sorption plays a significant role in the decolourisation process, and its contribution depends on both the type of support and the properties of the dye. Biomass on the PU carrier exhibited high sorption-desorption capacity, whilst PP was characterised by minimal desorption, which results from its hydrophobicity and lack of active binding sites. The highest desorption was observed for EB and CV, with the free fungal biomass of both strains releasing significantly more dye than the immobilised biomass, indicating a greater contribution of physical sorption in these systems. Immobilisation – particularly on sponge – led to more durable dye binding and lower desorption, which, according to the author, suggests the dominance of chemisorption and biodegradation processes, as well as a more stable dye-biomass-support interaction.

T. versicolor was more effective than *P. ostreatus* in removing RBBR; shaking further enhanced its enzymatic activity, enabling rapid (>90% in 24 h) dye oxidation. In the case of EB, both strains achieved high decolourisation, and the differences between static and dynamic conditions were minor, which, according to the PhD student, indicates that the degradation of azo dyes depends less on oxygenation than the breakdown of anthraquinone dyes.

An external carbon source clearly modulated the fungi's ability to degrade pigments (p. 76): *T. versicolor* achieved the highest efficiency, particularly when cultured under shaking conditions, which was thought to result from better oxygenation and stronger induction of ligninolytic enzymes, whereas *P. ostreatus* also decolourised the dyes, but to a much lesser extent, and the influence of sucrose as a carbon source did not improve efficiency compared to the standard medium, highlighting the importance of both oxygen conditions and the availability of appropriate nutrients for enzymatic activity. Doubling the glucose concentration revealed that excessively high levels of readily available carbon inhibit the activity of ligninolytic enzymes, resulting in lower decolourisation efficiency compared to a medium with standard glucose content. *T. versicolor* (CB8) continued to yield the best results, particularly under shaking conditions, whilst *P. ostreatus* (BWPH) responded slightly better to increased concentrations under static conditions, confirming that an optimal (rather than maximum) glucose concentration promotes the production of enzymes responsible for pigment degradation. The PhD student thus demonstrated that the effectiveness of decolourisation by white rot fungi is strongly dependent on the concentration of the external carbon source and optimal growth conditions, with the best results achieved under moderate carbon availability and good aeration. Excess carbon led to carbon catabolite repression (CCR), which limits the synthesis of ligninolytic enzymes; therefore, optimising carbon sources and oxygen conditions is crucial for maximising the efficiency of pigment removal.

Limited and excessive nitrogen availability clearly modulated the enzymatic activity of white rot fungi: the highest decolourisation efficiency for all dyes was achieved under nitrogen-deficient conditions, whilst media rich in nitrogen, particularly inorganic nitrogen, strongly inhibited degradation. The results unequivocally confirm the classical regulatory mechanism in white rot fungi, in which low nitrogen concentrations induce the synthesis of ligninolytic enzymes, whilst their excess leads to the inhibition of oxidative pathways, making the optimisation of nitrogen sources a key technological issue. Analysis of variance unequivocally confirmed (p. 85) that the nitrogen source was the strongest factor determining decolourisation efficiency, followed by the fungal species, and only then the carbon source, reflecting the key role of metabolic regulation in ligninolytic pathways. Tukey's post-hoc test showed that the highest RBBR removal efficiency was achieved by *T. versicolor* immobilised on a sponge in RM medium, and that immobilisation and aeration further enhanced performance, particularly in *P. ostreatus*, confirming the importance of selecting the species, carrier and medium composition for optimal decolourisation. Immobilisation of both species significantly increased the efficiency of dye degradation, outperforming both static cultures and those grown under shaking conditions, which, in the author's view, confirms that the structure of the PU carrier creates a stable environment conducive to high enzymatic activity and sustainable biodegradation of dyes. Lacc activity (p. 89) was clearly induced by all three dyes, and its level depended on the fungal species, dye type and growth conditions. The strongest enzymatic response was generated by *T. versicolor*, particularly in immobilised cultures, which directly translated into the highest degradation efficiency of RBBR and EB. *P. ostreatus* responded less strongly, though it exhibited a similar trend, dependent on oxygen conditions and the presence of the dye. The author concluded that immobilisation and good oxygenation strongly enhance laccase production, which remains a key factor in the degradation of dyes by white rot fungi. In contrast, MnP activity was strongly dependent on growth conditions and the presence of dyes, with *T. versicolor* achieving the highest levels in shaken cultures and the lowest in static ones. RBBR and EB most strongly induced early MnP production, which correlated well with the rapid rate of their decolourisation. *P. ostreatus* generally exhibited low MnP activity, with the exception of immobilised cultures treated with CV. The results confirm that high MnP activity, particularly in combination with Lacc, is crucial for the effective degradation of dyes, especially under conditions ensuring good oxygenation and metabolic stability. LiP activity in *T. versicolor* and *P. ostreatus* depended on the presence of dyes, incubation time and culture conditions. RBBR induced a rapid and intense increase in activity in CB8 (particularly under static conditions), while BWPH responded more slowly but with greater stability; these patterns correlated well with decolourisation efficiency. In the case of EB, a delayed induction of LiP and a gradual increase in activity were observed in BWPH, which also corresponded to a subsequent increase in dye degradation efficiency. The activity of ligninolytic enzymes did not correlate significantly with CV decolourisation. According to the PhD student, the results indicate that extracellular enzyme activity alone does not fully explain decolourisation efficiency, as non-enzymatic and intracellular processes also play a significant role. Systemic approaches, such as transcriptomic and proteomic analyses, are therefore necessary to gain a more precise understanding of the mechanisms of pigment degradation by fungi.

Transcriptome sequencing of *T. versicolor* (CB8) yielded high-quality, homogeneous data for all samples, providing a solid basis for analysing differences in gene expression under the influence of RBBR and EB dyes (p. 98). This study fills a significant gap, as detailed transcriptomic analyses of the degradation of these dyes by white rot fungi have been lacking, particularly in the context of intracellular detoxification pathways. The identification of differentially expressed genes enables the discovery of new mechanisms of xenobiotic metabolism and potential key genes for effective decolourisation, going beyond the classical approach based solely on extracellular enzymes. Satisfactory mapping levels (Fig. 37) confirmed that a suitable dataset had been obtained for further analyses, including the identification of differentially expressed genes, analysis of their ontology, and the identification of metabolic pathways. Following transcript assembly, gene reconstruction and the calculation of their expression levels, it was demonstrated that 3,623 genes showed no differences between samples – 10,939 genes were retained for further analysis. Analysis of DEGs between samples of mycelium exposed to RBBR and EB, and control samples, using more stringent criteria (FC > 2 and $p < 0.05$), allowed the number of identified differentially expressed genes to be narrowed down to 1,106 and 1,830, respectively. In both cases, strong downregulation predominated, while in the presence of RBBR, 318 genes showing overexpression were detected, which, in the author's opinion, indicates a distinctly different and intense transcriptional response of *T. versicolor* to both dyes. The expression histograms (Fig. 39) confirm that, under pigment stress conditions, the entire transcriptome shifts towards higher expression levels, particularly among genes with moderate activity. The author concludes that the fungus activates extensive metabolic, detoxification and stress networks, with the nature of the response depending on the chemical structure of the dye, reflecting different degradation strategies. Based on these results, the PhD student selected a set of the most strongly induced and repressed genes, which were subjected to ontological analysis (p. 108, 5.6.4) across three categories: molecular functions, biological processes and cellular components. Under conditions of exposure to RBBR, strong overexpression was observed of genes encoding oxidative enzymes (particularly CYP450), which, according to the PhD student, initiate the first stage of the attack on the anthraquinone structure of the dye, as well as numerous oxidoreductases and dehydrogenases involved in the detoxification of reactive intermediate metabolites. At the same time, increased expression of genes associated with glutathione-dependent detoxification (GST) and transporters indicates the activation of coordinated Phase II and III pathways of xenobiotic metabolism (neutralisation and elimination of products). Additional upregulation of shikimate pathway genes and mitochondrial enzymes suggests metabolic reprogramming of the fungus towards increased production of redox and energy precursors, essential for maintaining homeostasis during intense oxidative stress. Transcriptomic analysis of *T. versicolor* during EB. degradation revealed strong induction of genes associated with intracellular transport, energy metabolism and redox balance, including high (nearly 1200-fold!) overexpression of SNARE proteins and mitochondrial enzymes. The PhD student hypothesises that this results from intensification of vesicular transport and increased energy demand. At the same time, the expression of many regulatory and detoxification genes, such as GST, was inhibited, suggesting that the fungus favours pathways based on intensive redox reactions and the transport and sequestration of metabolites. The transcriptomic data were supplemented by proteomic analysis (p. 121, 5.7), revealing a varied number of proteins with altered expression depending on the type of dye, with RBBR inducing the strongest activation and EB the greatest repression of processes not directly related to degradation. According to the PhD student, this appears to confirm that the fungus reorganises its proteome, limiting growth-related functions in favour of oxidative, redox and transport enzymes, which are key to dye degradation. The author also highlights logical links between the transcriptome and the proteome, pointing to a consistent adaptation to stress following exposure to different classes of dyes. Bidirectional hierarchical cluster analysis revealed distinct proteomic profiles in *T. versicolor* depending on the type of dye, with RBBR appearing to elicit the most distinct response compared to the control. The analysis identified clusters of co-regulated enzymes (oxidoreductases, peroxidases and transporters) responsible for key stages of dye degradation, suggesting the simultaneous existence of conserved stress responses alongside dye-specific metabolic adaptations. The author suggests that *T. versicolor's* ability to flexibly adapt its proteome may be useful for optimising treatment processes. Proteomic analysis of *T. versicolor* under RBBR degradation conditions revealed strong activation of peroxidases and laccases, which appears to confirm the dominance of extracellular oxidative mechanisms in the breakdown of anthraquinone pigments. At the same time, proteins associated with oxidant detoxification were markedly elevated, whereas numerous enzymes involved in basal metabolism and biosynthesis were significantly repressed. The overall results confirm that *T. versicolor* prioritises highly reactive oxidative pathways for the effective decolourisation of RBBR at the expense of metabolic processes associated with proliferation. Under EB degradation conditions, *T. versicolor* responded with a reduction in protein levels, including key oxidoreductases; this, alongside the simultaneous expression of genes for redox enzymes and proteins associated with transport and compartmentalisation, may indicate a profound metabolic and structural reorganisation. The mechanism of dye degradation proposed by the PhD student involves, in sequence: extracellular oxidation of azo and anthraquinone structures by laccases and peroxidases, followed by the redox processing of reactive intermediate products within the cell by dehydrogenases, reductases and cytochrome P450, followed by their detoxification and compartmentalisation in the Golgi apparatus and in vacuoles, and finally the export of degraded metabolites via MFS transporters (as shown in Fig. 44). The PhD student notes that differences between the transcriptome and the proteome may stem mainly from the different timing of sampling and the biological context analysed (cell vs environment). Consequently the transcriptome reflects an early adaptive response, whereas the proteome reflects a late, functional set of degradative enzymes. The author points out that combining both approaches reveals complementary stages of *T. versicolor's* response to dyes, explaining the apparent discrepancy between mRNA levels and enzyme accumulation in the medium. She emphasises that this constitutes the first such detailed multi-omics analysis of the degradation of RBBR, EB and CV by *T. versicolor*, highlighting the innovative nature of the research.

Pure dyes exhibited high toxicity towards D. *magna* that increased with exposure time (p. 136, 5.8.1), which decreased significantly following decolourisation of the solution by both species. *T. versicolor* proved particularly effective in neutralising the dyes EB and CV, while *P. ostreatus* was most effective at reducing the toxicity of RBBR. The author emphasises that in every case, the activity of the fungi reduced the toxicity classification by at least one category, which indicates a real increase in environmental safety. The phytotoxicity of the tested dyes clearly depended on their chemical structure and the ability of individual fungal species to detoxify them. The results unequivocally confirm that white rot fungi are exceptionally effective biocatalysts in removing the toxicity of dyes. The PhD student demonstrated that both strains effectively decolourise RBBR and EB regardless of light conditions, suggesting that already developed biomass and an active enzymatic apparatus play a key role in dye removal. At the same time, subtle differences across different wavelengths indicate that light can modulate fungal metabolism and stabilise the activity of oxidising enzymes, which could potentially be exploited for optimisation.

The biomass under investigation exhibited a significantly higher sorption capacity and faster BG removal kinetics than most previously described biosorbents, which highlights its practical advantages. The results indicate that the initial phase of rapid sorption results from intense surface adsorption and high availability of binding sites, while the subsequent decrease in rate is associated with their gradual saturation. The study demonstrated that the CB8/S2-BS and BWPH/S2-BS variants exhibit high CV removal efficiency, particularly at lower initial concentrations, achieving over 60% reduction within the first few hours of the process. The observed differences in sorption efficiency between CV and BG result mainly from the different molecular weights of the dyes, which affect their diffusion and the availability of binding sites. PCA analysis (p. 152, 5.10.4, Figs. 51 and 52) unequivocally confirmed that contact time and initial dye concentration are key factors determining biosorption efficiency. The immobilised biomasses of both species consistently exhibited higher efficiency in removing BG and CV than their suspended forms, indicating a beneficial effect of the PU carrier matrix on the stability and availability of active sites. In case of both fungal species, immobilisation reduced sensitivity to dye toxicity and improved sorption kinetics, particularly under conditions of high dye loading. The author expresses the conviction that immobilisation constitutes a scalable and efficient solution for the treatment of dye-containing wastewater, enabling more predictable and effective dye removal under varied operating conditions. Autoclaved, immobilised *T. versicolor* biomass retained the ability to effectively remove BG and CV, whereas mycelial aggregates completely lost this ability, confirming the key role of the PU carrier matrix in stabilising the inactivated biosorbent (p. 155). Differences in mycelium structure resulting from growth conditions; the 'flattened' layer in static culture versus spherical aggregates (granules) under shaking conditions clearly determined the sorption capacity of the biomass (p. 157), which explains the greater efficiency of CB8 in removing BG and the lower efficacy of BWPH against CV. Maintaining a slightly acidic pH and moderate temperature proved crucial for maximising BG biosorption by the mycelia of both strains, as extremely alkaline or high-temperature conditions significantly reduced efficiency by destabilising the cell surface and limiting enzymatic activity. By characterising the nano- and microstructure of the mycelium matrix in greater detail, the PhD student revealed, with the aid of FT-IR spectroscopy, the involvement of key functional groups in the mycelium in the biosorption process, as evidenced by shifts and decreases in the intensity of the -OH and -NH bands following dye binding. Additional changes in the regions corresponding to C=O, C-N, C-O vibrations and aromatic signatures confirmed the presence of ionic, hydrogen, van der Waals and π-π interactions between the dyes and the biomass. Immobilisation on the PU matrix increased the intensity of bands in the protein and polysaccharide regions, indicating greater availability of functional groups and higher sorption capacity of the immobilised variants. SEM images confirmed that the PU sponge provides a highly porous, three-dimensional microstructure conducive to uniform fungal colonisation and effective biosorption of dyes. Both *T. versicolor* and *P. ostreatus* formed stable, extensive hyphal networks which, upon contact with BG and CV, exhibited distinct surface changes, such as smoothing, coating with a layer of dye, or partial pore blockage. A comparison of images before and after sorption unequivocally confirms the effective binding of dyes by and indicates that immobilisation on the sponge promotes greater stability of the mycelium as a biosorbent.

In the final section of the results discussion (p. 168, 5.11), the author discussed the purification experiment conducted in bioreactors. On a laboratory scale, *T. versicolor* demonstrated a high and sustained ability to decolourise RBBR, compared with the abiotic control. In the first two cycles, both the shaken culture and the immobilised biomass removed over 90% of the dye (Fig. 63), which, according to the doctoral Student, reflected strong enzymatic activity and the absence of an inhibitory effect from subsequent doses added to the inflow. The decline in efficiency in the third cycle suggested metabolic stress or the accumulation of metabolites; however, both systems still significantly outperformed the control. In the fourth cycle, stabilisation or partial recovery of activity was observed, and immobilisation on a sponge ensured comparable or higher performance, particularly in the initial stages of the bioreactor's operation.

In the final chapter (Future prospects of research, p. 173), the PhD candidate presents her views on the future direction of research, including: the development of continuous, modular bioreactors with immobilised biomass; process monitoring using molecular tools; the integration of fungal biomass reactors with existing treatment technologies; and research into the regeneration and reuse of immobilised biomass.

The PhD student summarised her dissertation with **15 conclusions** in the form of concise highlights, each with further elaboration. The research confirmed that *T. versicolor* is an exceptionally effective, stable and environmentally friendly method for removing synthetic dyes, and its performance can be significantly increased by optimisation of physicochemical parameters and immobilisation on low-cost carriers. Both *T. versicolor* and *P. ostreatus* demonstrated a broad spectrum of activity against various classes of dyes, high efficiency under high loads, and the ability to operate in bioreactor systems. Multi-omics analyses revealed that integrated intracellular detoxification pathways, including CYP450, oxidoreductases and membrane transporters, play a key role in degradation, rather than extracellular enzymes alone. Ecotoxicological tests confirmed that treatment with fungal biomass significantly reduces the toxicity of wastewater, confirming the environmental safety of the results obtained. The work as a whole provides a solid foundation for the design of sustainable, decentralised wastewater treatment systems based on white rot fungi.

The bibliography (compiled on pp. 175–195), to which the author refers in her dissertation, is cited in the order of appearance in the text, and comprises 303 entries. In this list, I also found three works co-authored by the PhD candidate (items 41, 143, 257), of which the first two were also listed in the bibliography in the introduction to the dissertation. The vast majority (≈ 90%) of the items selected by the PhD candidate are classic scientific articles. Although the oldest publication dates from 1938, and four from the 1980s and 1990s (though their citation was fully justified), the bibliography is very up-to-date, with a clear predominance of publications from the last five years (approx. 45%). These publications can be grouped into several thematic categories, including: wastewater treatment technologies for textile production, including physicochemical methods; the biodegradation of dyes by fungi and the adsorption of dyes on various substrates; issues in enzymology and biochemistry; genomics; fungal transcriptomics and proteomics, and the toxicology and environmental risk assessment of dyes.

4. Critical and discursive comments of an editorial and substantive nature

As a non-native speaker, unlike the author, the reviewer does not presume to assess the linguistic accuracy of the text. In my subjective opinion, the dissertation is written in clear language, the descriptions are lucid, and the arguments are logical, despite the methodological complexities and the multi-threaded nature of the work. However, given the sheer volume of the dissertation, and no doubt due to the rush and understandable fatigue of the PhD candidate during the final editing stage, it was difficult to avoid minor errors, which, in my view, do not affect the final assessment. I have taken the liberty of listing a few of them below, along with additional critical comments:

1. In my opinion, the discussions of the literature on multi-omics and toxicology should be consistently placed in separate subsections, if only because of the role they play in the rest of the work.
2. In publication [ref. 59], which the author cites when writing *"They can affect fish, amphibians, and invertebrates' ability to reproduce, grow, and behave..."*, the authors write rather generally about the impact of wastewater on fish, more in the context of the aforementioned reduction in DO concentration than toxicity. There is a certain amount of data on the impact of synthetic dyes on fish; observations include, amongst others, oxidative stress, hepatotoxicity, histological changes in the gills and

cardiovascular system, structural changes in tissues, lipid peroxidation, changes in the activity of antioxidant enzymes, and even genotoxicity (e.g. CR induced micronuclei and chromosomal aberrations). I was interested in the phenomenon of dye deposition in the lateral line of fish; I found no information regarding permanent saturation of this organ.

3. p. 7 – "*Lemna gibba, a common duckweed...*" - the paper cited as ref. 62 discusses the use of *Lemna gibba* for biomonitoring of wastewater from the textile industry – and as such is a correctly chosen source; however, the species mentioned is gibbous duckweed (g*ibbous duckweed*), not 'common duckweed' – i.e. *Lemna minor*.
4. p. 12. AOP – the term deep '*advanced oxidation*' does appear in the introduction, but there is no link to the abbreviation – as a rule, this should appear for the first time after it has been used in the text.
5. p. 57, Fig. 18. It says 'Photo by...' – in this case it is clearly a drawing, but the information about the photographer also appears unnecessarily in some of the other figures. In my view, even if the figure incorporates some minor photographic elements (e.g. a fragment of a sponge), it remains a drawing throughout. Perhaps this reflects a somewhat overly cautious approach to copyright – a self-drawn figure without additional notes implies that the entire content is the author's own creation.
6. In the Polish version of the abstract on page X, the phrase 'biologically sustainable alternative' appears; it should read 'sustainable alternative' or 'biological alternative'.
7. Throughout the paper, the author consistently uses the terms '*polypropylene dishwashing sponge*' and '*sponge'* (e.g. p. 27: '*Dishwasher-grade polypropylene'* [...] *Sponge - Commercial polyurethane*) to describe the carriers used in the studies for immobilising fungal biomass – in my opinion, a more scientific or technical term should be used – unless the author is referring to the source of supply for the bioreactors, or, for example, the use of waste for this purpose. Both materials used in the study, disregarding the functionality of their current form, are essentially examples of open-cell synthetic foam (PU foam, PP foam) formed as a result of the polymerisation of a given plastic (polyurethane or polyethylene) under specific conditions. Such materials can be described by many parameters – the most common is PPI (*pores per inch*), measured linearly. One could also attempt to determine the specific surface area experimentally or theoretically (expressed in cm^2/cm^3 or g), which is possible in various ways, e.g. based on the structural photographs that were, after all, taken. The author also provides certain characteristics of the biomass carriers that are key to describing the processes studied in the work in the context of dye properties, e.g. on p. 68. *"The hydrophobic characteristics of the polypropylene dishwasher likely restrict dye sorption efficacy*, and furthermore [...] *improved performance results from the high porosity, hydrophilicity, and increased surface area of the polyurethane substance* (p. 60), [...) *the polypropylene dishwasher does not have a tendency to absorb-desorb dye due to its hydrophobic nature".* I would encourage to take an interest in these parameters if the PhD student plans to conduct future research using carriers with a similar structure.
8. p. 43. – It appears that in the sentence "*For non-coding RNAs such as long intergenic non-coding RNAs (lincRNAs)* [**here**] *was removed using a Ribo-Zero RNA removal kit to enrich the RNA species of interest*", the term ***ribosomal RNA (rRNA)*** is missing.
9. p. 50. ImageJ as a non-commercial scientific programme should be cited, e.g. *Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods, 9(7), 671–675. doi:10.1038/nmeth.2089*
10. I found a few errors in the cited references: p. 175, item 13 – author is missing (*Anshuman N*.), p. 176, item 24 – authors missing (*Balasubramanian, C., Manickam, P*.), p. 177, item 33 – it appears there are no authors, p. 178, item 59 – authors missing (*Ishrat Bashir, F. A. Lone, Rouf Ahmad Bhat, Shafat A. Mir, Zubair A. Dar & Shakeel Ahmad Dar*), item 129: journal issue number missing *(15(1):6),* item 130: DOI number ending missing (*14548*), which directs the reader to a different article, p. 183, item 136 – authors missing (*M. Jureczko., W. Przystaś., M. Urbaniak., A. Banach-Wiśniewska., Ł. Stępień*), p. 192, item 256 – the paper by *Cuamatzi-Flores et al. 2024* is listed twice in the bibliography (including item 197), p. 165, item 300 – an author appears to be missing (*Tepe, Ö*?)
11. In taxonomic nomenclature species names are written in lowercase, e.g. *Agaricus* ***bisporus****, Ceriporia* ***lacerata****, Diaporthe* ***schini***, and in the absence of a species designation, *Trametes sp.* – incorrect entries occurred mainly in the bibliography.

At the same time, my role as a reviewer allows me to ask a few questions, which I ask the doctoral candidate to take as a sign of curiosity and a spark for discussion, which should not be missing at the defence.

1. Toxicity tests, particularly in environmental risk assessment, require the use of model organisms representing all trophic levels – including decomposers (most commonly bacteria). As an expert in the reactions of filamentous fungi, is the PhD student familiar with toxicity tests using fungi as model organisms, or are the species studied in the dissertation suitable for this purpose in practice? In the PhD student's opinion, what does, or should the procedure for such a study look like?
2. p. 48. *'...using standard fresh water'* – does this refer to standardised water (in which case, what is its composition?) or tap water?
3. p. 50. Did the phytotoxicity test methodology also include observations of the loss of living biomass in the test plants, e.g. the reduction in leaf area affected by chlorosis? Was such a phenomenon observed?
4. In the case of transcriptomic data, particularly at the first stage of their processing, should we be talking about genes or rather expression tags? How would you define the differences between these terms?
5. On p. 44, we read: *"For Gene Ontology (GO) and KEGG [...] pathway analyses, differentially expressed genes (DEGs) with an absolute fold change ≥2 |...]"*. In RNA-seq analysis, are we dealing with absolute or relative quantification of gene copy numbers?
6. In the final section of the dissertation, the author writes that an important direction for future research will be the monitoring of gene expression under operational conditions. In this regard, the question arises as to whether, and if so which genes, the PhD student would propose for monitoring and controlling the process in the reactor using, for example, quantitative PCR (qPCR)?
7. A picture emerges from the multi-omics data of an organism that carries out the wastewater decolourisation process under conditions of severe stress, restructuring its architecture at various levels of cellular organisation with the aim of survival rather than biomass expansion. Is it possible, then, to envisage a technological system for treating textile wastewater that operates continuously, maintaining the biomass, or rather a reactor supplemented with portions of biomass produced in a preliminary reactor, and, for example, removed every few treatment cycles for desorption? Which form of biomass would be most suitable in such a system? How long would it take to cultivate sufficient biomass for wastewater treatment on an industrial scale?

5. Summary and conclusion

Ms Ruchi Manishkumar Upadhyay is applying for the award of a doctoral degree in the field of engineering and technical sciences, in the discipline of environmental engineering, mining and power engineering. I have read the doctoral dissertation submitted to me for assessment with great interest and rate it very highly. I would like to emphasise once again that the very scope of the analyses required to achieve the objectives set by the doctoral candidate constituted an ambitious challenge – also for the reviewer. The dissertation presents an original solution to the problem, particularly in the section concerning the multi-omics analysis of fungal biomass involved in the dye degradation process. The implementation of modern research methods, including those from the field of molecular biology, in environmental engineering is becoming increasingly necessary to effectively optimise technological parameters in biological reactors. At the same time, the dissertation retains a character that allows it to be classified within the discipline, and all the results achieved are considered in the context of their future application in full-scale engineering systems.

I hereby conclude that the doctoral dissertation by Ms Ruchi Manishkumar Upadhyay, MSc, entitled "*Determination of the mechanism and optimisation of the conditions for the removal of colored aromatic compounds by selected Basidiomycota*" meets the requirements defined in the Act of 2 July 2018 - Law on Higher Education and Science (Journal of Laws of 2024, item 1571, as amended) and, in view of the above, I request the Discipline Council for Environmental Engineering, Mining and Power Engineering at the Silesian University of Technology to admit Ruchi Manishkumar Upadhyay, M.Sc. to a public defence.

At the same time, due to the high scientific and cognitive value of the dissertation, I request the Discipline Council to award the dissertation a distinction.